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Use of Cholera Toxin B as a Vaccine Adjuvant Activates Antigen Presenting Cells and Stimulates Production of Pro-Inflammatory Cytokines

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Use of Cholera Toxin B as a vaccine adjuvant activates antigen presenting cells and stimulates production of pro-inflammatory cytokines

By

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I would like to thank my family, my mentor Dr. Bitsaktsis, and my boyfriend Jon Godek

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Abstract

Francisella tularensis is an intracellular pathogen that has been classified as a category "A" bioterrorism agent by the Centers For Disease Control. To date, there is no approved vaccine to provide protection against this pathogen. Previous *in vivo* studies with mice have shown that a mucosally targeted vaccine preparation of inactivated *F. tularensis* (*iFt*) adjuvanted with Cholera toxin "B" (CTB), successfully granted full protection against a less virulent strain (*FT LVS*) of the bacterium and provided partial protection against a more virulent strain (SchuS4). However, the mechanisms of this protection are not fully understood. In this present study, an *in vitro* system was utilized to further elucidate the mechanisms that drive protection against lethal F. tularensis challenge in *iFt+CTB* mucosally immunized mice. Specifically, the focus was directed towards determining the effects of *iFt+CTB* on macrophages, the common host of *F. tularensis*, and their ability to present antigen to naïve T-cells, express costimulatory molecules, and produce pro-inflammatory cytokines. We found that RAW264.7 cells, a murine macrophage cell line, responded to treatment with *iFt+CTB* by an increased secretion of the pro-inflammatory cytokines IL-6 and TNF-α. It was also determined that treatment with *iFt+CTB* up-regulated the expression of TLR4 on the macrophage cell surface. The effects of *iFt+CTB* treatment were shown to increase the expression of both costimulatory molecules B7.1 and B7.2 on the macrophage cell surface. Furthermore, we found that *iFt+CTB* enhanced the ability of macrophages to present antigen to a *FT-specific* T-cell hybridoma cell line. These findings allow us to elucidate in part, the mechanisms of protection against *F. tularensis* challenge in *iFt+CTB* immunized mice.

Introduction

1.1 Introduction to Vaccines

Vaccines are one of the most successful achievements of modern medicine. Many pathogens that have plagued mankind throughout history, such as polio, measles, small pox, and diptheria, have been controlled or eradicated through successful vaccination programs. Since Edward Jenner pioneered the first vaccine in 1796 using cowpox material to protect against smallpox, vaccination has truly become a modern marvel of human ingenuity and cleverness.

There are many methods that have been successfully used to grant protection from various bacteria, viruses, and toxins. Immunization can be granted through either passive or active means, in a natural or, as is the case of a vaccine, an artificial manner (Clem, 2011). Passive immunization occurs with the transfer of preformed antibodies to an unimmunized individual, conferring a rapid, effective, but temporary immunity. This type of immunity can be acquired naturally through the passage of maternal antibodies to a fetus through the placenta, and later through breast milk. Artificial passive immunity, on the other hand, is transmitted through delivering functional preformed antibodies to an unimmunized individual, such as through the use of antivenin serums that contain preformed antibodies designed to target a specific venom (Clem, 2011). Active immunity stimulates the adaptive immune system, subsequently conferring long-term memory against a pathogen, through the generation of long-lived memory T-cells and B-cells. These cells, known as lymphocytes, “remember” a specific previously encountered antigen and are able to respond more effectively and rapidly compared to the primary challenge. Natural active immunity is achieved through the process of infection with the actual pathogen followed by its successful clearance from the body. Vaccines are able to

grant active immunity by an artificial means, wherein they mimic the stimulation necessary to elicit an adaptive immune response, thereby generating immunological memory, without producing disease (Clem, 2011).

1.2 The Systemic Immune System and the Generation of Immunological Memory

Successful vaccines must generate immunological memory (Clem, 2011). A vaccine must safely provoke a deliberate and strong immune response, thereby activating the appropriate branch of the adaptive immune system, resulting in the generation of memory (Kindt et al., 2007). However, in most cases it is the innate branch of the immune system that first encounters the antigen.

The innate immune system is the first line of defense against invading pathogens, specifically through its anatomical and chemical barriers, as well as through a collection of cells and molecules that mediate inflammation. The cells of innate immunity recognize foreign molecular patterns, by utilizing their pattern recognition receptors (PRRs). PRRs, although not specific to a particular pathogen, can recognize pathogen-associated molecular patterns (PAMPs) (Clem, 2011). Recognition of PAMPS by PRRs leads to the development of an inflammatory response, which entails complement activation, opsonization, cytokine release, and phagocyte activation. Macrophages and dendritic cells are types of innate cells that, upon activation, have the ability to recruit the assistance of the adaptive immune system via antigen presentation. Antigen presentation is crucial for the successful activation of, what is perhaps the most pivotal cell of the adaptive response, the CD4+ T-helper cell.

On their own, T-cells are unable to recognize free antigens. They depend upon the efforts of antigen presenting cells (APCs) to process and present antigens directly to them, in association with the major histocompatibility complex (MHC). CD4+ T-helper cells only recognize antigens presented on MHC class II molecules, which are found exclusively on these antigen presenting cells (Clem, 2011). Antigen presenting cells (APCs) provide the vital bridge to the adaptive immune system and the generation of immunological memory. There are only three professional APCs that possess the necessary surface molecules to activate naïve CD4+ T-cells: Macrophages, B-cells, and dendritic cells. These three APCs not only express MHC class II, but they also express the costimulatory molecules, B7-1 (CD80) and B7-2 (CD86), both of which provide the required second signal for T-cell activation (Kindt et al., 2007). APCs both process and make direct contact with antigens in one of two ways. The first, is via an exogenous route that allows antigens from outside the cells to be phagocytosed and presented via MHC class II. The second, is via an endogenous route where antigens from within the cell, such as viral proteins, are processed and presented to CD8+ T-cells via MHC class I. All nucleated cells have the ability to present antigens, via the endogenous route, on a class I MHC molecule, indicating intracellular infection and directing the cytotoxicity of CD8+ T-cells. However, it is only the professional APCs that have the unique ability to process antigens through the exogenous route, presenting on MHC class II molecules which are recognized by CD4+ T-cells. After the APC successfully activates the naïve T-cell, the lymphocyte begins to proliferate and differentiate into memory and effector cells.

1.3 Vaccination Strategies and Constraints

Many successful vaccines, including the Sabin polio vaccine, are composed of live, attenuated, whole microorganisms (Kindt et al., 2007). Live attenuated vaccines cause a strong immune response that will result in lifelong immunity. However, there are safety concerns with delivery of live pathogens into a healthy individual. As was the case with the Sabin vaccine, there is a risk of attenuated pathogens reverting back to the virulent form, causing disease after vaccination. Inactivated, or killed whole microorganism vaccines provide a safer alternative than that of live attenuated. Unfortunately, they do not stimulate a robust cellular immune response, and thus typically require booster shots. Other effective vaccines have been developed that utilize inactivated toxins, known as toxoids. The diphtheria vaccine, for example, is composed of inactivated versions of bacterial exotoxins that cause production of neutralizing antibodies which, in turn, protect against the active form of the toxin. Subunit vaccines exploit specific antigenic fragments of a pathogen in an effort to induce an immune response, as has been successfully accomplished with the hepatitis B vaccine. Although subunit vaccines offer safer protection, they often prove tremendously difficult to determine a protective antigenic subunit, and then subsequently develop a vaccine.

Vaccines, in general, have seen an enormous amount of success in the past century. However, conventional vaccine strategies have been pushed to their limitations, and have proven to offer very limited protection against many pathogens, including HIV, tuberculosis, and malaria, making it necessary to research more innovative immunizing strategies (Yuki and Kiyono, 2003). The majority of successful vaccines to date are administered by injection, intramuscularly or subcutaneously, conferring protective systemic immunity. Many pathogens however, gain

access to the body through mucosal surfaces via ingestion, inhalation, or sexual contact. Traditional injectable vaccines fail to induce protective immunity at the mucosal surfaces, leaving this critical route of entry vulnerable. Interestingly, mucosal vaccines, which are administered at the mucosal surfaces, have been shown to generate systemic protection in addition to mucosal immunity (Yuki and Kiyono, 2003). Mucosal vaccine systems may ultimately prove superior by conferring this dual immunity, while additionally deterring pathogens at the initial site of invasion. Unfortunately, most protein antigens that are administered mucosally are very poor immunogens. Furthermore, to their own detriment, they can induce immune tolerance, the opposite of the desired response (Yuki and Kiyono, 2003). Development of mucosal vaccines has long been hindered by this phenomenon. Fortunately, promising research has been focused on the development of safe mucosal adjuvants.

An added constraint in most vaccinations used today, both injectable and mucosal, is that they elicit a strictly humoral protective response from the host (De Pascalis, 2012). Through a variety of mechanisms, antibodies are proficient at controlling or limiting many types of infection, however due to their inability to cross the plasma membrane their usefulness is confined to outside the cell. Upon infection with an intracellular pathogen, such as *Fracisella tularensis*, the value of antibody protection becomes diminished and cell-mediated immunity takes a primary role in eradicating the invading microbe. The tactics of infection displayed by intracellular parasites warrant the design of a vaccine that stimulates a cell-mediated immune response. In addition to the activation of the specific branch of the immune system, a vaccine that targets the initial site of infection would farther boost its potential for success. Most pathogens gain access to the body through mucosal surfaces; for this reason, the mucosa has

evolved an almost entirely separate immune system that has yet to be thoroughly exploited by vaccine developers.

1.4 The Mucosal Immune System

Mucosal surfaces of the gut, airway, and urogenital tract provide an interface between the body's tightly regulated interior environment and that of the continuously changing external environment (Kraehenbuhl and Neutra, 1999). Mucosal membranes are consistently on the frontline of exposure to potentially dangerous microorganisms, as well as a vast array of benign normal flora. It is due to their inherent vulnerability that mucosal surfaces have developed a localized component of the immune system. Specialized mucosal lymphoid tissues uniquely uptake and process antigens, produce distinct immunoglobulins, and activate effector cells in a manner that is nearly independent from the systemic immune system (Kraehenbuhl and Neutra, 1999). The mucosal immune system can effectively be divided into distinct inductive and effector sites (MacDonald et al., 2013) (see figure 1).

These inductive sites collectively known as mucosa-associated lymphoid tissue (MALT) are comprised in a similar manner to lymph nodes. MALT houses B-cell follicles, interfollicular T-cell regions, and an assortment of antigen-presenting cells. Notably, MALT structures lack afferent lymphatic vessels and must sample antigens directly from the mucosal surfaces. Antigen sampling takes place through unique regions on the mucosal epithelium known as follicle-associated epithelium (FAE) which covers the underlying mucosal lymphoid tissue. There are three methods of antigen sampling that occurs in these inductive regions of MALT. First, involves specialized epithelial cells known as microfold (M) cells which are located

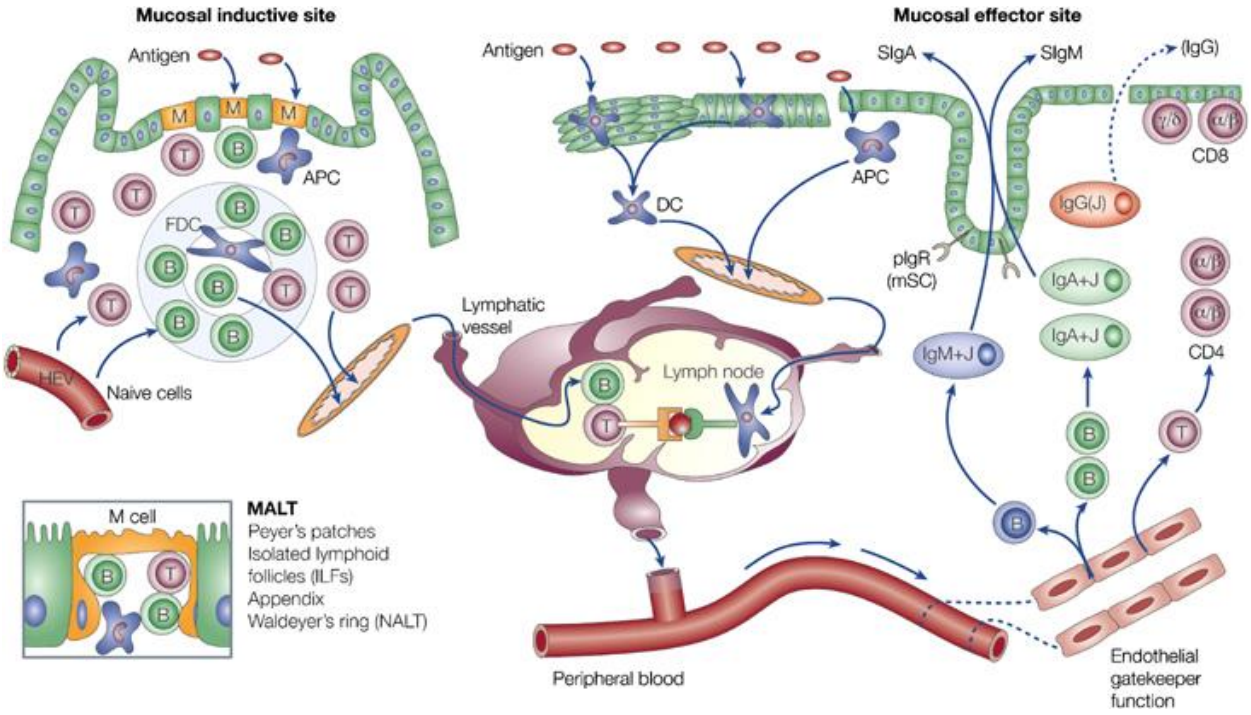

Figure 1

Overview of the human mucosal immune system. Reprinted by permission from Macmillan Publishers Ltd: Mucosal Immunology, 1(1), 32, Brandtzaeg, P., Kiyono, H., Pabst, R., & Rusell, M. (2008). Terminology: nomenclature of mucosa-associated lymphoid tissue.

exclusively in the FAE. M cells have the unique function of transporting antigenic material from the lumen to dendritic cells (DCs), macrophages, and lymphocytes of the MALT waiting below the epithelium (MacDonald et al., 2013). Besides M cell sampling, DCs have the ability to reach their dendrites in between epithelial cells to directly capture antigens in the lumen. Thirdly, immune complexes can transcytose through mucosal epithelial cells by means of the Fc neonatal receptor. Once an antigen reaches the lymphoid tissue it may be presented by various APCs to local lymphocytes, phagocytosed by resident macrophages, or brought to lymph nodes via dendritic cells. Once in the possession of an antigen, DCs may activate T-cells immediately in the subepithelial dome or they may migrate to regional lymph nodes. Lymphocytes activated in MALT structures migrate through the lymphatic system and home in on mucosal effector sites through the expression of various integrins and cell adhesion molecules (CAMs) (MacDonald et al., 2013).

Mucosal effector sites are often populated by activated lymphocytes, macrophages, dendritic cells, and granulocytes. Here, MALT derived mucosal plasma cells are the body's largest population of antibody secreting cells; predominantly IgA. These plasma cells produce enough dimeric secretory IgA (SIgA) to effectively protect the majority of mucosal surfaces. Mucosal macrophages are present through the entirety of the mucosa, where they actively neutralize microorganisms, inert material, and apoptotic cells by phagocytosis. While the mucosal immune system can be quite proficient at controlling a multitude of infections, it should be noted that this system is not independent. It is closely associated with the systemic immune system.

1.5 Francisella tularensis

The causative agent of tularemia, *Francisella tularensis*, is a small gram-negative bacterium that exists as a pleomorphic coccobacillus (Wong and Shapiro, 1999). It is a facultative intracellular parasite that can survive and multiply within host macrophages, as well as within other cell types (Harvey et al., 2007). There are two subspecies of *Francisella tularensis*, *tularensis* (biovar A) and *holarctica* (biovar B) (Bitsaktsis et al., 2009). Of these subspecies, biovar A strains, such as *SchuS4*, prove to be the most virulent in humans as well as other mammals. The first human cases of tularemia were reported in 1907, and were found to be linked to the contact of wild animals (Wong and Shapiro, 1999). This rural disease, which came to be known as "Rabbit Fever", is most frequently contracted by hunters, trappers, veterinarians, livestock handlers, and those who are frequently exposed to a variety of different species of mammals. Contact with the bacterium's preferred reservoir, wild rabbits of the genus: Sylvilagus, as well as bites from arthropod vectors, such as the tick, are the most common causes of infection. *F. tularensis* is an immensely durable organism. Its occurrence is found naturally within many different species, including mammals, arthropods, and avians, as well as abiotic components of the environment, such as water, mud, and animal feces. In the United States, tularemia is endemic to Arkansas, Missouri, and Oklahoma, with the highest incidence of infection occurring during the summer months (Harvey et al., 2007).

The Centers for Disease Control has classified *F.tularensis* as a category "A" bioterrorism agent due to its ease of dissemination, its potential to substantially impact public health and induce widespread panic, and the requirement of special action that would need to be undertaken by public health officials, if such an event were to occur (Bioterrorism, www.bt.cdc.gov). In

addition to this, *F. tularensis* has become exceptionally well known for its potential to be wielded as a weapon in biological warfare. During the 1930s and 1940s, numerous outbreaks of waterborne tularemia in both Europe and the Soviet Union incited large epidemics that brought widespread publicity to the potential threat of this organism if used as a biological weapon (Dennis et al., 2001). During World War II, biological warfare research facilities in the United States, Japan, and the Soviet Union began experimenting with the use of *F. tularensis* for military purposes. The United States has since admitted to the development of weaponized *F. tularensis* disseminating aerosols, during the 1950s and 1960s. In fact, for such purposes, aerosol deployment of this organism would be the ideal delivery system. It is one of the most infectious pathogenic bacteria known today, requiring the inhalation of as little as ten organisms to cause disease (Dennis et al., 2001). The victims of such an attack would be readily stricken with a severe, and often fatal, pneumonic form of tularemia. Once the pathogen has been inhaled, it is able to quickly gain access to the body through the mucosal surfaces of the respiratory tract, making its threat, and subsequent impact to public health and welfare all the more severe. The development of a mucosal vaccine designed to combat *F. tularensis* is the primary, if not ideal strategy that must be undertaken to protect against pneumonic tularemia is to be successful. It is through the development of such a vaccine that protection at the primary site of exposure to the pathogen can be achieved.

1.6 Vaccine adjuvants

During vaccine development it is imperative to elicit an immune response to the protective antigen administered, but very few molecules are immunogenic when they contact mucosal

surfaces (Rappuoli et al., 1999). Adjuvants are substances that when mixed with, or conjugated to, an antigen enhance the immunogenicity of that antigen. Generally, adjuvants cause the up-regulation of costimulatory molecules, prolong antigen presence, increase local inflammation, and stimulate proliferation of lymphocytes (Kindt et al., 2007). The most powerful mucosal immunogens known to date are cholera toxin (CT), which we will focus on in this study, as well as *Escherichia coli* heat-labile enterotoxin (LT) (Rappuoli et al., 1999). These molecules cause the severe diarrhea seen in cholera and traveler's diarrhea. The mucosal immune system is able to recognize these toxins as a threat and mount a strong immune response against them. CT and LT are very similar in structure, both of which consist of two subunits: a pentameric "B" oligomer, which allows the toxin to bind to receptors on the surface of eukaryotic cells. The other, an enzymatically active "A" subunit is responsible for toxicity. The toxic effects caused by the intact cholera toxin molecule initiate when the cholera toxin B subunit (CTB) binds to the ganglioside GM1, believed to be a major toxin receptor, which is located on the surface of a host epithelial cell (Rappuoli et al., 1999). The "A" subunit is anchored to the "B" pentamer by means of a trypsin sensitive loop, and a long alpha helix whose C-terminus enters the central cavity of the "B" pentamer. After CTB binds to the GM1 ganglioside, the host cell internalizes the toxin into vesicles; the holotoxin is then cleaved by proteases into its subunits within the Golgi apparatus. The now enzymatically active "A" subunit translocates to the cytosol where it acts as an ADP ribosylase on the alpha subunit of regulatory heterotrimeric G proteins, thus inducing covalent adenylate cyclase activation. This persistent activation leads to abnormal levels of the second messenger, cAMP, accumulating within the cell, which is related to the induction of the profuse fluid expulsion seen in these debilitating diarrheal diseases.

The ability of enterotoxin molecules to generate such an intense mucosal response, in an environment that tolerates most potential antigens, makes them of much interest to mucosal vaccine research. The use of toxin molecules as adjuvants has shown promising results. Nevertheless, they must be rendered safe by some method of separation of adjuvanticity from toxicity. Past studies have shown that a nasal influenza vaccine employing an enterotoxin adjuvant produced a significant number of cases of Bell's palsy in vaccinees (Rhee and Lee, 2012). It has been suggested that the enterotoxin binds with high affinity to the GM1 ganglioside receptors of the olfactory nerves and begins to build up in the brain producing this undesired side effect. Not all mechanisms of enterotoxin adjuvanticity are understood and must be elucidated before additional clinical trials become a possibility.

A significant amount of research has been aimed at the immune modulating effects of cholera toxin and its subunits. In the past, studies have cultured macrophage cell lines in the presence of intact cholera toxin, reporting enhanced antigen presentation which ultimately leads to elevated T-cell proliferation (Bromander et al., 1991). It was also determined that macrophages cultured with CT produced elevated levels of the pro-inflammtory cytokine IL-1. The adjuvant activity seen with intact cholera toxin was originally thought to be associated with its toxicity. However, it was not until further studies had been undertaken that the pentameric "B" subunit was determined to have no enzymatic activity and can function as an effective adjuvant alone (Wu and Russell, 1998). Initially, the adjuvant effects of CTB were suspected to have been the result of intact CT contamination in commercially available CTB stocks. In order to verify that CTB alone could act as an adjuvant, an in vivo study involving mice was conducted at the University of Alabama. There, the adjuvant effects of highly purified recombinant CTB to the

commercially available formula of CTB, and to a mixture of CTB/CT were compared. The results demonstrated that intranasal administration of pure CTB functioned quite successfully as an adjuvant, thus increasing the mucosal and systemic antibody response in treated mice (Wu and Russell, 1998).

1.7 Use of CTB as a Vaccine Adjuvant in vitro

One mechanism of adjuvanticity of CTB can be attributed to its efficiency as a mucosal carrier molecule, wherein antigens conjugated to CTB are effectively delivered to APCs through high affinity binding to the GM1 ganglioside on APC surfaces (George-Chandy et al., 2001). In addition to antigen delivery, CTB has also been shown to enhance the ability of antigen presentation by APCs. *In vitro* studies have shown that antigens conjugated to CTB greatly reduce the amount of antigen needed to stimulate a proliferative T-cell response and allow macrophages to function as efficient APCs to naïve T-cells. It was determined that CTB conjugated antigens enhanced T-cell proliferation, largely due in large to increased levels of IL-12, IFN-gamma, and increased expression of the costimulatory molecules CD40 and CD86 on the surfaces of APCs (George-Chandy et al., 2001). Furthermore, previous studies have focused on the intracellular effects of CTB on antigen presenting cells (De Pascalis, 2012). It has since been determined that tyrosine kinases play a significant role in the CTB induced transactivation of transcription factor NF-κB during macrophage activation. CTB induced NF-κB transactivation led to increased expression of CD86, MHC II, CD54, CD14, and CD40 in bone marrow-derived macrophages. The same study established that macrophages grown in vitro increased

secretion of the pro-inflammatory cytokine IL-6 in response to treatment with CTB (De Pascalis, 2012).

1.8 Use of CTB as a Vaccine Adjuvant in vivo

A recent *in vivo* study with mice, conducted by Bitsaktsis et al., has shown that CTB acts as an effective adjuvant in experimental mucosal vaccine preparations against *F. tularensis* (Bitsaktsis et al., 2009). Remarkably, these results have shown that when administered intranasally, CTB simply mixed with inactivated *F. tularensis LVS* (*iFt*) conferred 100% protection against a lethal challenge of *Francisella tularensis* live vaccine strain (*FT LVS*, biovar B) and partial protection against the more virulent *SchuS4* (Biovar A) strain is provided. Perhaps more interestingly, this protection is specifically in the form of an interferon-gamma dependent Th1 response, which completely negated the need for antibodies. This is a novel development in the use if CTB as an adjuvant, due to the fact that the adjuvant was merely mixed with and not conjugated to the antigen, but also on the account that the prior consensus was that CTB elicits a strictly Th2 antibody dependent response when administered intranasally (Wu and Russell, 1998);(Bromander et al., 1991);(Lycke, 1997). Additionally, the elicitation of cell-mediated protection by a vaccine is exciting for the potential of future developments of vaccinations against intracellular pathogens.

In this present study, an *in vitro* system was utilized to further elucidate the mechanisms that drive protection against lethal *FT LVS* challenge in *iFt+CTB* immunized mice. Specifically, the focus was directed towards determining the effects of *iFt+CTB* on macrophages, the common host of *F. tularensis*, and their ability to present antigen to naïve T-cells, express costimulatory

molecules, and produce pro-inflammatory cytokines. For this purpose, populations of the murine macrophage cell line, RAW 264.7, were exposed to experimental treatments of *iFt+CTB*. We found that RAW 264.7 macrophages responded to treatment with *iFt+CTB* by an increased secretion of the pro-inflammatory cytokines IL-6 and TNF-α. It was also determined that treatment with *iFt+CTB* up-regulated the expression of TLR4 on the macrophage cell surface, while treatment with *iFt* alone resulted in down-regulation of this receptor. The effects of *iFt+CTB* treatment were shown to increase the expression of both costimulatory molecules B7.1 and B7.2 on the macrophage cell surface. Furthermore, we found that *iFt+CTB* enhanced the ability of RAW 264.7 cells to present antigen to a *FT-specific* T-cell hybridoma cell line. These findings allow us to elucidate in part, the mechanisms of protection against *FT LVS* challenge in *iFt+CTB* immunized mice. Treatment with this experimental vaccine may ultimately prove the importance of macrophages in vaccine mediated *F. tularensis* protection.

Materials and Methods

2.1 Bacteria

F. tularensis live vaccine strain (*FT LVS*) organisms were provided by Dr. Edmund Gosselin (Albany Medical College, Albany, NY). *F. tularensis* organisms were grown in Mueller-Hinton broth supplemented with 2% IsovitaleX™ (Fisher Scientific) enrichment to a concentration of $2.6x10^9$CFU/ml at 37°C. Live samples of *F. tularensis* were stored in liquid nitrogen at -80°C in supplemented Mueller-Hinton broth.

2.2 Immunogen

Inactivated *F. tularensis LVS* (iFt) was used as the immunogen for these experiments. *F. tularensis* bacteria were grown to a concentration of $2.6x10^9$CFU/ml in supplemented Mueller-Hinton broth at 37°C. 25ml of live bacteria were centrifuged and the pellet was resuspended in 25ml of 2% paraformaldehyde (PFA) and sterile PBS solution. Bacteria were fixed by an incubation of 90 minutes in the PFA solution at room temperature while shaking. After fixation, inactivated *F. tularensis* were washed three times in sterile PBS. To confirm inactivation, 100ul of iFt ($2.6x10^8$ CFU) were plated on chocolate agar plates (Fisher Scientific) and observed for growth for seven days. Inactivated *F.tularensis* was stored in PBS at -20°C.

2.3 Cell Culture

The murine monocyte/macrophage cell line RAW 264.7 was provided by Dr. Allan Blake (Seton Hall University Biology Department, South Orange, NJ). Cells were grown in RPMI 1640 media

(Lonza) supplemented with 2mM L-Glutamine (Lonza), 10% fetal bovine serum (Lonza), and 1x penicillin/ streptomycin (VWR International).

The *FT-specific* T-cell hybridoma (FT256D10) cell line was provided by Dr. Edmund Gosselin (Albany medical College, Albany, NY). Cells were grown in RPMI 1640 media (Lonza) supplemented with 2mM L-Glutamine (Lonza), 10% fetal bovine serum (Lonza), 1x penicillin/streptomycin (VWR International), 1mM sodium pyruvate (Sigma), 1% MEM non-essential amino acids (Sigma), 50µM 2-mercaptoethanol (Sigma), and 1% Hygromycin (Sigma). All cells were cultured in a humidified atmosphere of 5% CO2 at 37C.

2.4 Cytokine Measurement

RAW 264.7 cells were plated in 12 well plates at a concentration of $5x10^5$ cells per well and allowed to incubate at 37°C in 5% CO2 for four hours. Cells were then treated with either PBS, CTB (Sigma)(1µg/ml or 5µg/ml diluted in PBS), *iFt* ($1x10^5$ CFU/well diluted in PBS), or a combination of *iFt* ($1x10^5$ CFU/well diluted in PBS) and CTB (1µg/ml or 5µg/ml diluted in PBS). Supernatants were collected at 2 hours, 12 hours, 24 hours, or 48 hours after treatment, depending on the nature of the cytokine being tested and stored at -20°C until analysis. Cytokines were later measured by ELISA using ELISA Max kits (BioLegend) specific for IFN-γ, TNF-α, IL-10, IL-4 and IL-6. All ELISA analyses were performed according to the manufacturer's instructions.

2.5 Determination of cell surface markers

RAW 264.7 cells were plated in 12 well plates at a concentration of $5x10^5$ cells per well and allowed to incubate at 37°C in 5% CO2 for four hours. Cells were then treated with either PBS, CTB (1µg/ml or 0.5µg/ml diluted in PBS), *iFt* ($1x10^5$ CFU/well diluted in PBS), or a combination of *iFt* ($1x10^5$ CFU/well diluted in PBS) and CTB (1µg/ml or 0.5µg/ml diluted in PBS). Cells were collected after 24 hours of incubation with treatments and immediately labeled using the following antibodies from BioLegend: Pacific Blue™ conjugated-anti-mouse CD86 (cat#105021), Alexa Fluor® 488 conjugated-anti-mouse I-A/I-E (polymorphic determinant on $I\text{-}A^d$ MHC class II) (cat#107615), phycoerythrin (PE) conjugated-anti-mouse CD80 (cat#104707), Alexa Fluor® 647 conjugated-anti-mouse/human CD282 (TLR2) (cat# 121809), Phycoerythrin (PE) conjugated-anti-mouse CD284 (TLR4), Pacific Blue™ conjugated-anti-mouse/human CD11b (cat#101223), phycoerythrin (PE) conjugated-anti-mouse/human CD11b (cat#101207), and Alexa Fluor®488 conjugated-anti-mouse/human CD11b. Once labeled, cells were fixed in a solution of 2% paraformaldehyde and FACS buffer (PBS, 5% FBS, and 0.1% sodium azide). A flow cytometer (Macs Quant) was used to analyze fluorescent emission of tagged fluorphores.

2.6 Antigen Presentation assay

RAW 264.7 cells were plated in 12 well plates at a concentration of $5x10^5$ cells per well and allowed to incubate at 37C in 5% CO2 for four hours. Cells were then treated with either PBS, CTB (1µg/ml or 0.5µg/ml diluted in PBS), *iFt* ($1x10^5$ CFU/well diluted in PBS), or a combination of *iFt* ($1x10^5$ CFU/well diluted in PBS) and CTB (1µg/ml or 0.5ug/ml diluted in PBS). RAW 264.7 cells were incubated with experimental vaccine treatments for 24 hours. The following day, all

media and treatments were removed from the wells. The RAW 264.7 cells were then exposed to the immunogen diluted in FT256D10 media. This experiment was first performed using live FT LVS as the immunogen, then repeated using inactivated *FT LVS* (*iFt*) as the immunogen. $1x10^6$ CFU/well of live *FT LVS* or *iFt* (diluted in FT256D10 media) was added and allowed to incubate for 15 minutes before addition of T-cells. Next, $2.5x10^5$cell/well of the *FT-specific* T-cell hybridoma (FT256D10) are added and allowed to incubate for 24 hours. Three additional control groups were plated as well: macrophages with only *FT LVS* or *iFt*, macrophages with only T-cells, and T-cells with only *FT LVS* or *iFt*. Supernatants are collected and stored at -20°C. The T-cell hybridoma (FT256D10) secretes IL-5 upon activation. Supernatants were analyzed for the cytokine IL-5 by ELISA using a BioLegend ELISA Max kit as per manufacturer's instructions.

2.7 Statistical Analysis

Statistical data for cytokine analysis was generated using paired Student's t-test comparing concentrations of each experimental group. GraphPad Prizm 4 analysis software was used. Flow cytometeric data was analyzed using FlowJo software.

Results

3.1 CTB enhances the ability of the iFt immunogen to elicit secretion of pro-inflammatory cytokines from RAW264.7 macrophages

A successful vaccine must invoke a strong immune repose in order to ultimately generate the desired memory cells. Therefore, we wanted to determine a cytokine profile for the RAW 264.7 cells cultures with experimental vaccine treatments. The inflammatory response elicited from the RAW 264.7 cells suggests that the macrophages were in fact stimulated by *iFt+CTB* as compared to cells treated with *iFt* alone. This could have indicated in the very least a protective primary response and is an encouraging result in the design of this vaccine. ELISA analysis was performed for the pro-inflammatory cytokines IL-6, IFN-ɣ, and TNF-α. Analysis of the IL-6 ELISA data (fig 2A and B) indicated that groups treated with CTB and *iFt+CTB* had an increased secretion of IL-6 when compared to groups that were not exposed to CTB. Furthermore, groups treated with *iFt+CTB* had an increased secretion of IL-6 when compared to groups treated with only CTB. This data suggests that CTB may work synergistically with *iFt* to increase the secretion of IL-6. Similarly, analysis of the TNF-α ELISA data (fig 2C and D) showed an increase of secretion of TNF-α in groups treated with CTB and *iFt+CTB* as compared to groups not exposed to CTB. However, this data proves that CTB alone drove the secretion of TNF-α and no synergistic effect was observed in groups treated with *iFt+CTB*. ELISA analysis did not detect the secretion of IFN-ɣ in any experimental group. The anti-inflammatory cytokines IL-4 and IL-10 were also measured by ELISA analysis, but were not detected in any of the experimental groups (data not shown). From these results, we can conclude that the production of these

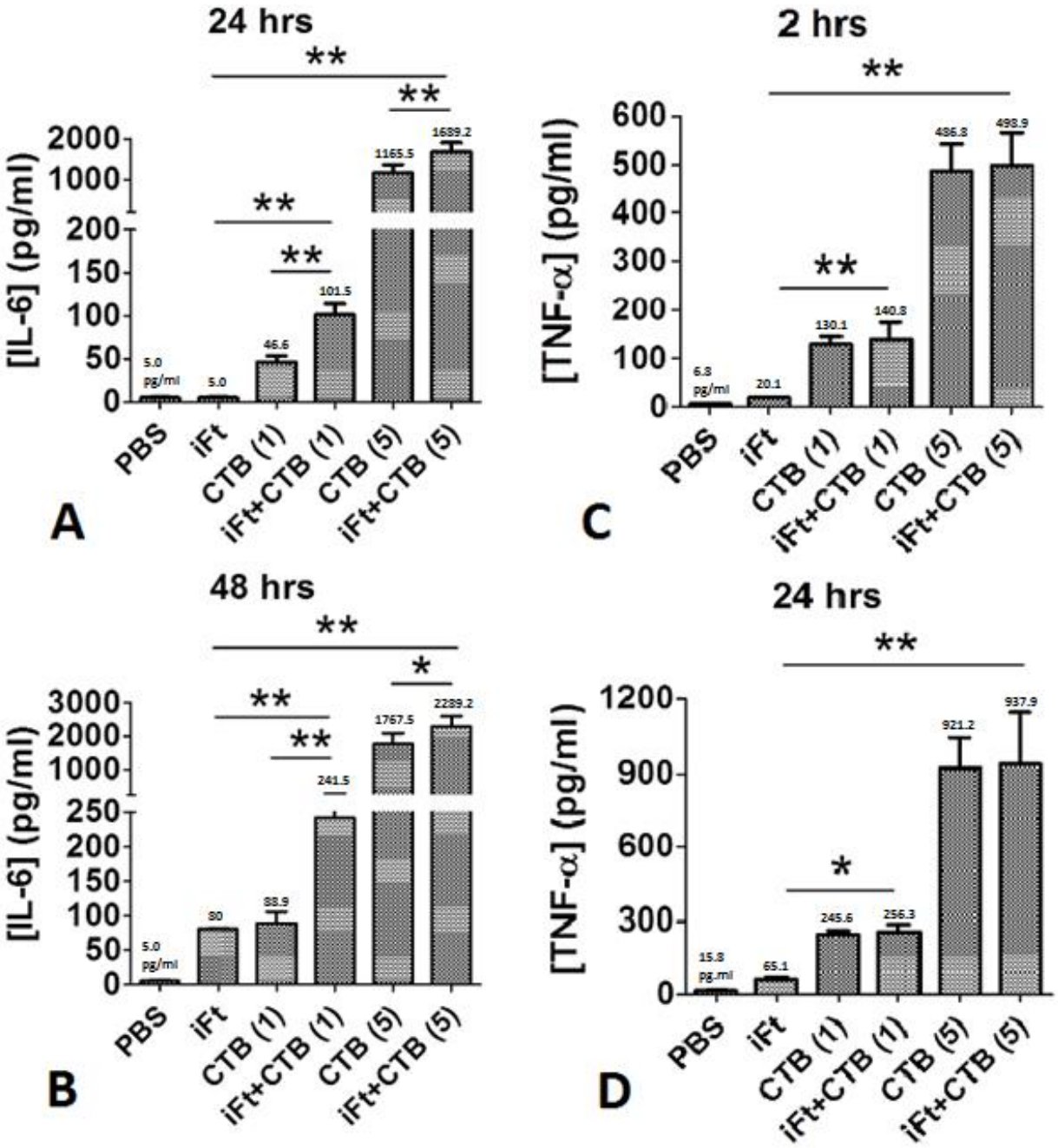

Figure 2. *iFt+CTB* has a pro-inflammatory effect on RAW 264.7 cells. Increased levels of the pro-inflammatory cytokines IL-6 (A and B) and TNFα (C and D) were detected by ELISA analysis in RAW264.7 cells exposed to *iFt+CTB* as compared to groups treated with PBS, *iFt* alone, or CTB alone. (*, $p < 0.05$ and **, $p < 0.01$, n=3)

pro-inflammatory cytokines indicates that the macrophages are activated and capable of driving an inflammatory response.

3.2 CTB used as an adjuvant up-regulates expression of TLR4 on RAW264.7 cells

Toll-like receptor 4 (TLR4) is a pattern recognition receptor on the surface of macrophages. TLR4 recognizes Lipopolysaccharide (LPS), a component in the cell wall of bacteria. Ligation of Toll-like receptors, including TLR4, is an essential signal for macrophage activation. Through flow cytometric analysis data, we have determined that the presence of CTB in the treatment group has led to the up-regulation of TLR4 (figure 3). Specifically, we have seen a 37.2% and 44.8% increase in TLR4 expression after 24 hours of incubation in the *iFt+CTB* treatment groups as compared to the control (PBS) and *iFt* alone groups respectively (figure 3A,B,C, and G). These results are further intensified after 48 hours of incubation, with an observed increase of 59.2% and 67.9% more TLR4 expression when compared to the negative control and *iFt* groups respectively (Figure 3D,E,F, and G). This data also indicates a down-regulation of TLR4 expression in cells treated with *iFt* alone when compared to the negative control group. After 24 hours of incubation, there was a 7.6% decrease in TLR4 expression in groups treated with *iFt* alone. Again these results were intensified after 48 hours of incubation, with an observed 8.7% decrease of TLR4 expression in the cells treated with *iFt* alone.

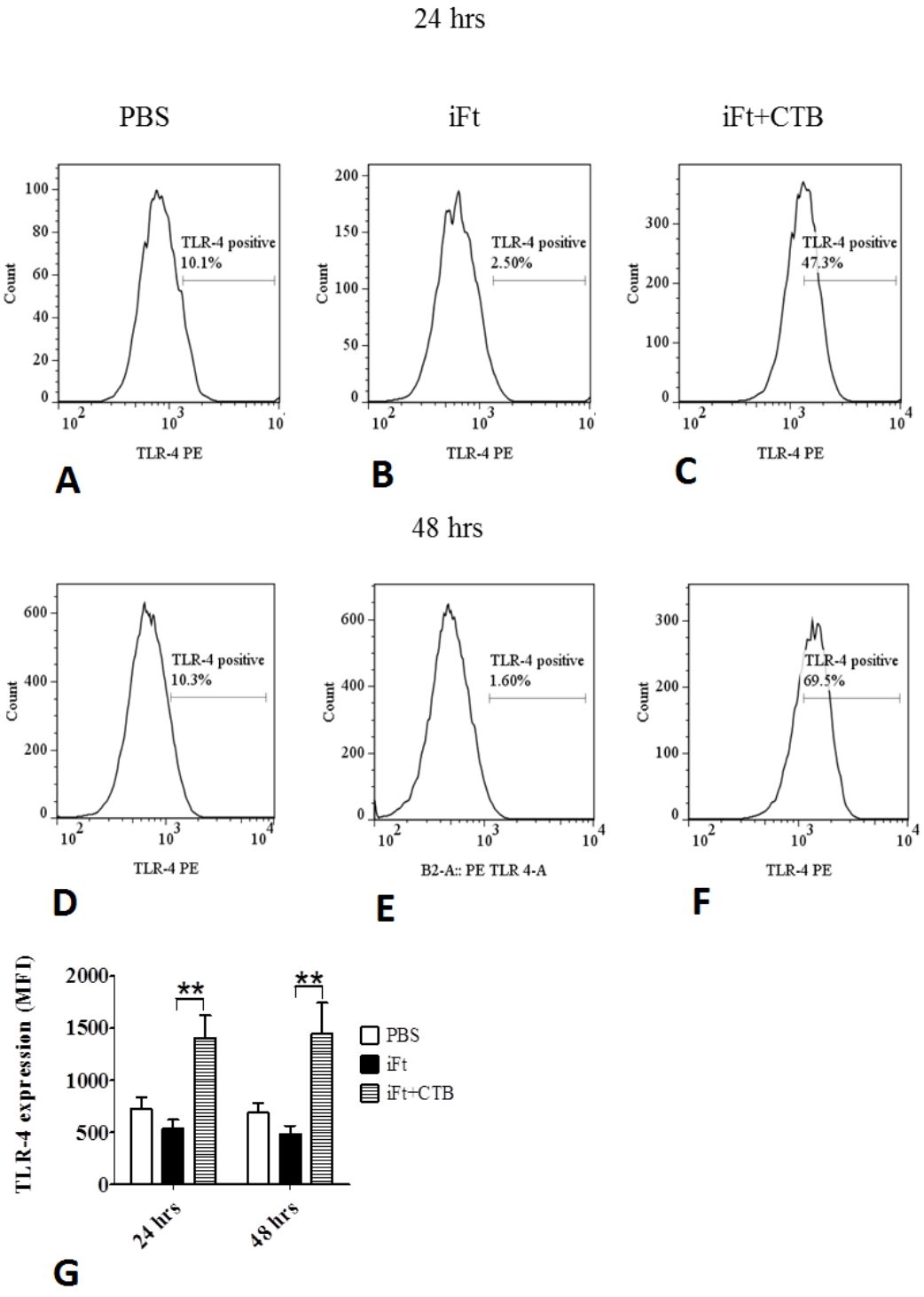

Figure 3. *iFt+CTB* up-regulates TLR4 expression on RAW 264.7 cells. Flow cytometric analysis detected enhanced expression of TLR4 in cells treated with a combination of *iFt* and CTB (C, F and G) when compared to treatment with PBS (A and D) or *iFt* alone (B and E). (*, $p < 0.05$ and **, $p < 0.01$, n=2)

3.3 CTB up-regulates the costimulatory cell surface molecules B7.1 (CD80) and B7.2 (CD86) on the surface of RAW 264.7 cells

Unstimulated macrophages express low levels of the costimulatory cell surface molecules B7.1 and B7.2. These molecules provide the necessary second signal during activation of a naïve T-cell. B7.1 and B7.2 were labeled with a flourophore conjugated antibody followed by flow cytometric analysis. The levels of B7.1/2 were determined individually along with the presence of MHC class II, the required molecule which provides the first signal during T-cell activation. Cells were collected after 24 and 48 hours of incubation in experimental treatments. After 24 hours of incubation, it was determined that cells treated with *iFt+CTB* (1µg/ml and 5µg/ml) had a significant increase in expression of B7.1 on their cell surface (figure 4A and C). The cells treated with a combination of *iFt+CTB* (at a concentration of 1µg/ml or 5µg/ml) had the most significant increase of B7.1 expression, with 68.5% and 75.1% of cells expressing B7.1 and MHC class II as compared to the control (PBS) (4.15% expressing both molecules) and the group treated with *iFt* alone (4.91% expressing both molecules). Similar results were observed after 48 hours of incubation in experimental treatments (figure 4B and C).The cells treated with *iFt+CTB* (at a concentration of 1µg/ml or 5µg/ml) had the most significant increase of B7.1 expression, with 52.0% and 68.9% of cells expressing B7.1 and MHC class II as compared to the percentages of the cells expressing both molecules in the control (PBS) (2.81%) and *iFt* alone treated groups (3.0%). The flow cytometric analysis revealed similar results for the expression of B7.2. After 24 hours of incubation in experimental treatments (figure 5A and C), we observed 14.1% and 18.4% of cells with dual B7.2/MHC class II expression in groups treated

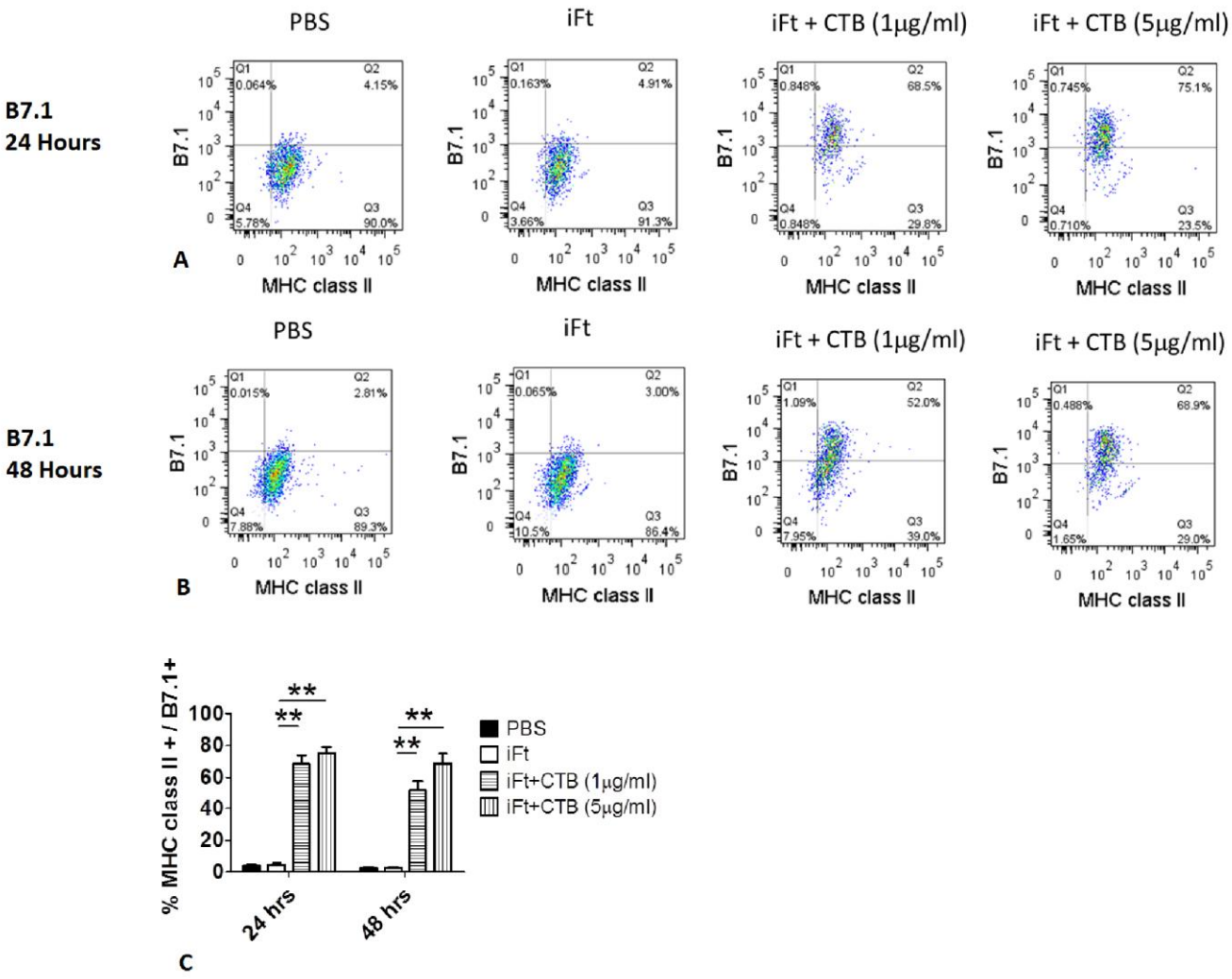

Figure 4. *IFt+CTB* up-regulates the expression of B7.1 on RAW 264.7 cells. Expression levels of the costimulatory molecule B7.1 is up-regulated in groups treated with *iFt+CTB* at 24 hours (A and C) and 48 hours (B and C) of incubation when compared to groups treated with PBS or *iFt* alone. (*, $p < 0.05$ and **, $p < 0.01$, n=2)

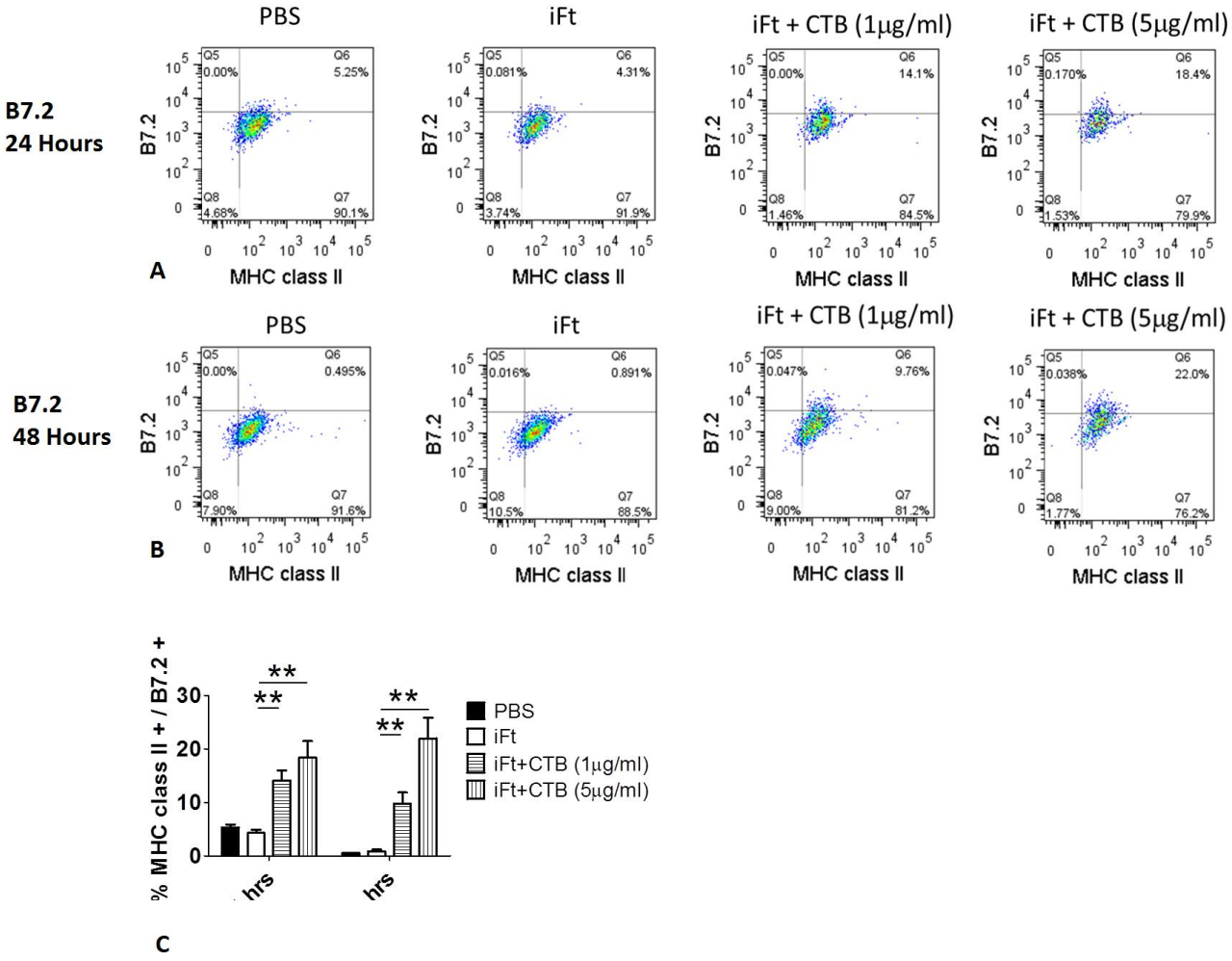

Figure 5. *IFt+CTB* up-regulates the expression of B7.2 on RAW 264.7 cells**.** Expression levels of the costimulatory molecule B7.2 is up-regulated in groups treated with a combination of *iFt* and CTB at 24 hours (A and C) and 48 hours (B and C) of incubation when compared to groups treated with PBS or *iFt* alone. (*, $p < 0.05$ and **, $p < 0.01$, n=2)

with *iFt+CTB*, at a concentration of 1ug/ml and 5ug/ml respectively, as compared to 5.25% dual expression in the control and 4.31% dual expression in the *iFt* alone treatment group. This same trend is further seen after 48 hours of incubation in experimental treatments (figure 5B and C), we observed 9.76% and 22.0% of cells with dual B7.2/MHC class II expression in groups treated with *iFt+CTB*, at a concentration of 1µg/ml and 5µg/ml respectively, as compared to 0.495% dual expression in the control and 0.891% dual expression in the *iFt* treatment group. Though the levels of expression are not as pronounced as what was seen with B7.1 expression there was still a statistically significant difference between the *iFt+CTB* treated groups as compared to the control (PBS) and the groups treated with *iFt* alone. It can be concluded that these essential costimulatory molecules are up-regulated by treatment with a combination of *iFt+CTB*. Furthermore, we can deduce that CTB and *iFt* are working synergistically to increase B7.1/2 expression due to the fact that cells treated with *iFt* alone or CTB alone (data not shown) did not have a significant increase in expression levels. The presence of B7.1/2 on the surface of the macrophage may enhance the cell's ability to present antigen to a naïve T-cell.

3.4 Pretreatment of Raw 264.7 macrophages with iFt+CTB enhances antigen presentation in vitro

After 24 hours of exposure to the same experimental vaccine treatments as previously described on page 18, Raw264.7 cells were cultured with either live *FT LVS* or *iFt* as the antigen. The ability of these murine macrophages to present the antigen and subsequently activate *FT-specific* hybridoma T-cells (FT256D10) was analyzed. FT256D10 T-cells secrete IL-5 in response to activation. ELISA analysis was used to determine the IL-5 concentration in treatment groups.

When cells were cultured with *iFt* as the antigen, enhanced IL-5 production (>10 pg/ml) was observed in cells pretreated with *iFt+CTB* as compared with cells pretreated with *iFt* alone (figure 6B). This amplified secretion of IL-5 indicated that there was improved antigen presentation occurring in these cells treated with *iFt+CTB.*

The experiment was repeated using live *FT LVS* as the antigen. Low levels of IL-5 (< 10 pg/ml) were observed in *iFt+CTB* and *iFt* alone pretreatment groups (figure 6A). The lack of IL-5 secretion indicates a lack of successful antigen presentation occurring in all cells cultured with live *FT LVS*.

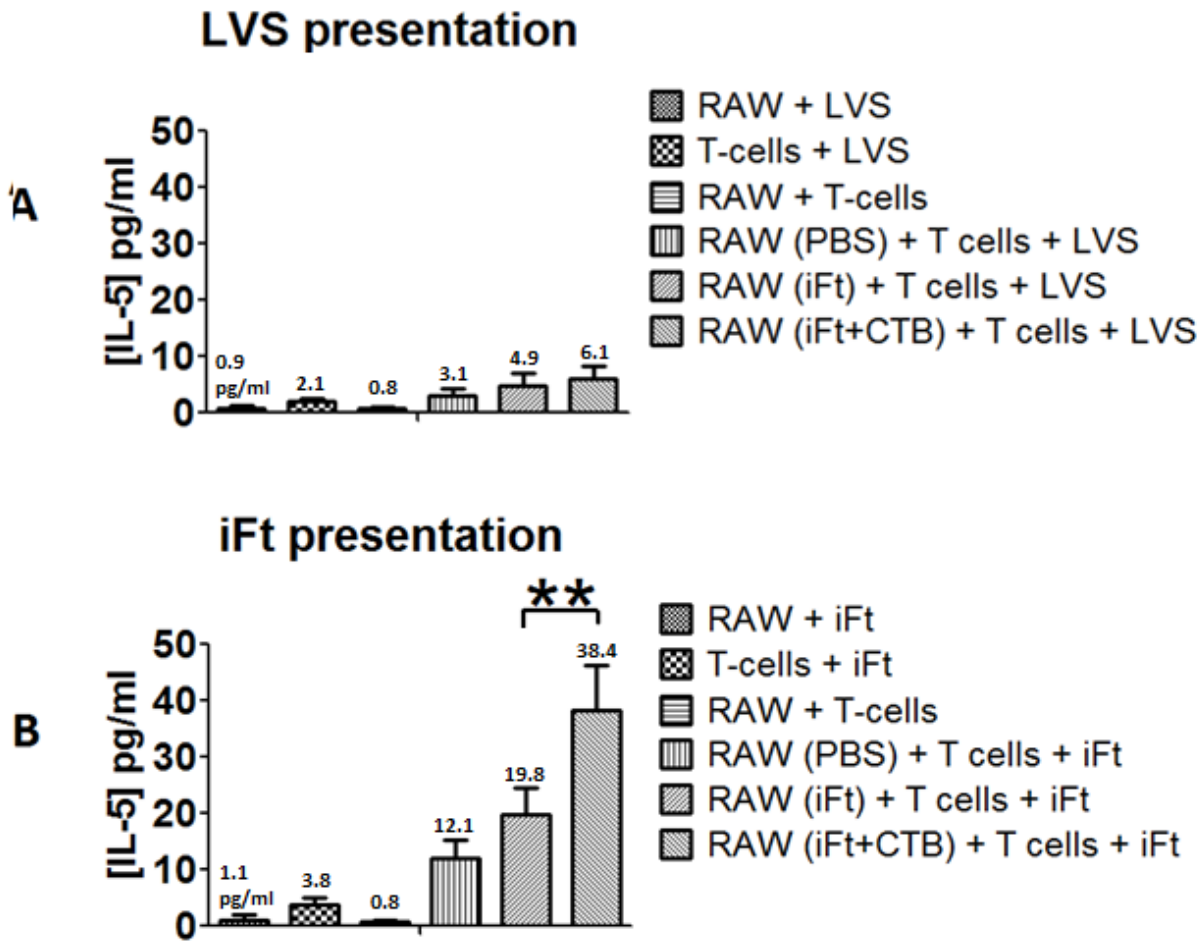

Figure 6. *iFt+CTB* enhances the ability of RAW264.7 cells to present antigen to a *FT specific* T-cell hybridoma cell line when *iFt* is used as the antigen. When the antigen presentation assay was ran using live *FT LVS* (A) as the antigen, low levels of IL-5 were secreted in all control and experimental treatment groups. However, when the antigen presentation assay was repeated using *iFt* (B) as the antigen, enhanced secretion on IL-5 was detected in experimental groups by ELISA analysis. Furthermore, when *iFt* was used as the antigen there was a significant increase in IL-5 production in cells treated with *iFt+CTB.* (*, $p < 0.05$ and **, $p < 0.01$, n=3)

Discussion

Recent *in vivo* studies have demonstrated that mice immunized with a combination of *iFt* and CTB show a 100% survival rate following infection with a lethal challenge of *FT LVS* and partial protection against the more virulent *SchuS4* strain (Bitsaktsis et al., 2009). This same study went on to further prove that this protection was IFN-ɣ dependent and could completely negate the need for antibodies. In the present study we aimed to further elucidate the innate mechanisms generated by this vaccine platform after exposure to the same experimental vaccine treatment *in vitro*.

The pro-inflammatory cytokine production observed by the *iFt+CTB* treated RAW 264.7 cells yielded promising results on the vaccine's potential effects on macrophages. Secretion of these pro-inflammatory cytokines indicates that these macrophages are attempting to induce inflammation, confirming that the desired immune response is occurring in reaction to the vaccine treatment (Kindt et al., 2007). *In vivo*, the acute-phase inflammatory response provides rapid protection after tissue injury and, more importantly for this study, is crucial for clearing invading bacteria. The healthy acute inflammatory response is the first line of defense after exposure to a pathogen, it should be quickly activated, clear the pathogen, and cease. On the local level, inflammation is characterized by redness, swelling, pain, heat, and loss of function. Immediately at the site of injury/infection, vasodilation and increased vascular permeability occurs, allowing for greater blood flow and leakage of fluids from the blood. The inflammatory process is a positive feedback driven chain reaction event. The edema in the affected tissue causes multiple enzymatic cascades to initiate, including the bradykinin, fibirin, and plasmin systems, further triggering vascular permeability as well as complement activation (Kindt et al.,

2007). Complement system activation will induce mast cell degranulation, releasing histamine an extremely powerful mediator of inflammation. After a few hours, these vascular changes will provoke neutrophils to extravasate out of the blood vessels and into the tissue, where they begin to phagocytose pathogens, release further mediators of inflammation, and secrete chemokines. These chemokines will attract activated macrophages to the site of infection, about 5-6 hours after initial injury/infection. Activated macrophages have an increased ability to phagocytose and kill pathogens, as well as secrete three characteristic cytokines, TNF-α, IL-1, and IL-6, all of which further propagate the inflammatory response. TNF-α is an extremely powerful mediator of inflammation, which displays multiple actions during the acute phase response. Increased expression of E-selectin, an endothelial cell adhesion molecule that specifically adheres to adhesion molecules on neutrophils, is observed in the presence of TNF-α. TNF-α also promotes the further secretion of chemokines from macrophages and endothelial cells, which recruit added neutrophils to the site (Kindt et al., 2007). Moreover, TNF-α along with IFN-ɣ activate naïve macrophages and neutrophils, resulting in enhanced phagocytosis and secretion of lytic enzymes in the affected tissue. IL-6, at the site of infection, induces the release of IL-8, another chemokine that attracts neutrophils, basophils, and some subpopulations of lymphocytes. These changes induced by the inflammatory response at the local level result in the accumulation of lymphocytes, monocytes, neutrophils, eosinophils, basophils, and mast cells at the site of injury/infection, which are successful in clearing the invading pathogen in most cases. While the inflammatory response occurs at the local level, a systemic inflammatory response takes place. The systemic inflammatory response is characterized by fever, hormone secretion, namely ACTH and hydrocortisone, increased

production of white blood cells, and emission of acute phase proteins by the liver. Most of these systemic changes are similarly due to the combined effects of TNF-α, IL-6, and IL-1.

The secretion of IL-6 in response to treatment with CTB has been previously documented in bone marrow derived macrophages (De Pascalis, 2012). Therefore, the increased secretion of IL-6 observed by *iFt+CTB* treated RAW264.7 cells was expected and could have signaled that the desired immune response to the experimental vaccine had indeed occurred. However, there is a marked increase of IL-6 secretion in cells treated with a combination of *iFt+CTB* when compared with cells treated with CTB alone. This signifies that *iFt+CTB* are working synergistically to increase the amount of IL-6 produced, and that this level of secretion was not simply a response to the presence of CTB. This is a desired effect in an adjuvant, for an adjuvant alone should not induce an immune response (Bitsaktsis et al., 2009).

The increased secretion of TNF-α also indicated that the desired immune response was occurring upon treatment with CTB. However the data established that the response was driven by the CTB alone, and was not correlated to the presence of *iFt*.

It is important to note, that only activated macrophages are able to secrete pro-inflammatory cytokines, which raises the question of RAW 264.7 cell activation. Macrophages are classically activated by two signals; TLR ligation followed by the action IFN-ɣ (Mosser, 2003). In the present study, ELISA analysis has shown that there is no detectable IFN-ɣ present in the treatment wells (data not shown). This may prove a method of adjuvanticity exerted by the CTB, which may itself be acting as a second signal to activate macrophages negating the need for IFN-ɣ.

Interestingly, this data (Figure 3B, E, and G) has indicated a down-regulation of TLR4 in the *iFt* alone treated cells. *Francisella tularensis* is known to have immunosuppressing properties, previous studies have established that macrophages infected with the live *FT Schu4* strain do experience TLR4 down-regulation; however this has not been documented after exposure to *iFt* (Butchar et al., 2008). The continued ability of *iFt* to down-regulate TLR4 is significant and the mechanisms by which this occurs have yet to be determined. The up-regulation of TLR4 seen in the *iFt+CTB* treated groups is one of the adjuvant effects conferred by CTB, and may account for the synergistic effect observed in the increased IL-6 secretion of *iFt+CTB* treated groups. Increased expression of TLR4 would allow for a higher number of receptors present on the surface of the cell to be ligated with LPS, and thus increase the likelihood for macrophage activation and subsequently increase IL-6 secretion.

Macrophages are typically unable to activate naïve T-cells. This is a protective mechanism due to the fact that macrophages continuously scavenge dead, or senescent host cells and the presentation of a self-antigen to T-cells could result in autoimmune disease (Janeway and Travers, 1994). Though they are the most inept APC they can efficiently and reliably present antigen after exposure to microbial elements in the proper cytokine milieu. Macrophages must become activated in order to process and present antigen to T-cells and express the mandatory costimulatory molecules. As mentioned above, macrophages classically need two signals to activate (Mosser, 2003). The first signal is the essential cytokine IFN-gamma, usually secreted by T-lymphocytes, this cytokine primes the macrophage for activation, but does not activate the cell itself. The second signal is tumor necrosis factor (TNF), which is typically endogenously produced by the macrophage itself, following ligation of various Toll-like receptors by assorted

microbial products. Only activated macrophages are able to produce pro-immune response cytokines such as TNF-α, IL-12, IL-6, and IL-1 and generate toxic antimicrobial oxygen and nitrogen species such as NO and O2-. On the contrary, macrophages exposed to IL-4 or glucocorticoids assume an "alternatively activated" phenotype. These alternatively activated macrophages act much differently than the classically activated macrophage. These cells do not make NO or O2- and are unable to clear intracellular pathogens. Performing more as immune-regulatory cells, they are not efficient at presenting antigen to naïve T-cells and may in fact inhibit T-cell proliferation. Recent studies have shown that live *FT LVS* has the ability to shift classically activated macrophages to the alternatively activated phenotype *in vitro* as well as *in vivo* (Shirey et al., 2008). This may account for the lack of antigen presentation seen during the antigen presentation assay when live FT LVS was cultured with the cells. Furthermore, when *iFt* was used as the antigen, enhanced antigen presentation was seen in all experimental groups demonstrating that the live bacterium could be negatively influencing the macrophage's ability to present antigen. In addition to the enhanced antigen presentation seen in all experimental groups, there was a significant increase in antigen presentation of macrophages treated with *iFt+CTB*. It is plausible to conclude that this improved presentation may be due to the observed increased expression of the two costimulatory molecules B7.1 and B7.2 in response to treatment with *iFt+CTB*. It also reinforces the notion that vaccine preparations against *Francisella tularensis* should only utilize inactivated organisms or subunits, and not live attenuated preparations. The increased expression of B7.1/2 detected along with the presence of the MHC class II molecule on the surface of these macrophages confirms that they are equipped and able to activate naïve T-cells and consequently generate T-memory cells.

The ability of *iFt+CTB* to activate macrophages, up-regulate key cell surface molecules, provoke the secretion of pro-inflammatory cytokines, and improve antigen presentation, may lead to the hypothesis that it may be the enhanced activity of the macrophage that is contributing to the 100% survival rate previously granted by *iFt+CTB* in *in vivo*. However, the ability of live *FT LVS* to negatively impact antigen presentation by macrophages *in vitro,* leads to the conclusion that their involvement in vaccine granted protection is strictly innate. The ability of live *FT LVS* to counteract the *iFt+CTB* granted ability for macrophages to successfully present antigen to *FT-specific* T-cells clarifies that it is not primarily the macrophage that generates a protective adaptive immune response.

This leads us to hypothesize that the dendritic cell (DC), the most proficient antigen presenting cell, may be key to the previously observed protection *in vivo*. Currently studies are being conducted to elucidate the potential effects of *iFt+CTB* immunization on dendritic cells and to further understand the mechanisms that mediate protection in this vaccine platform.

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